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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
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JAN 20 1975

Application for Research Grant
(Use extra pages as needed)

Date: Jan. 15/75

1. Principal Investigator (give title and degrees):

   James Travis, Ph.D, Associate Professor

2. Institution & address:

   University of Georgia
   Athens, Georgia 30602

3. Department(s) where research will be done or collaboration provided:

   Department of Biochemistry

4. Short title of study:

   Biochemistry of Chronic Obstructive Lung Disease

5. Proposed starting date: July 1, 1975
6. Estimated time to complete: Three years
7. Brief description of specific research aims:

   (see attached sheet)

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7. SPECIFIC RESEARCH AIMS

The process of pancreatic secretion of proteolytic enzymes is under double control in that these enzymes are a) all produced as inactive zymogens which cannot, therefore, degrade pancreatic tissue and b) they are secreted together with large quantities of a pancreatic protease inhibitor (Kazal secretory inhibitor). Thus, proteolysis is limited to the gut area. However, during acute attacks of pancreatitis, proteolysis is no longer controlled and not only are zymogens activated but also the secretory inhibitor is rapidly destroyed.

The proposed project essentially attempts to determine whether the parallel regulation of proteolysis in tissue is under control by serum factors. Particularly, we are asking the importance of α-1-PI, since it represents over 90% of the total inhibitory activity of serum or plasma. Because α-1-PI deficiency correlates strongly with the development of emphysema such a hypothesis is tempting. However, more questions have really been raised than have been answered. For example, does α-1-PI exert any control over major proteolytic reactions, such as occur in the coagulation and fibrinolytic systems? Can proteases secreted from phagocytizing cells actually overwhelm the normal regulatory system and degrade normal tissue? Are these proteolytic enzymes secreted in response to particular stimuli which do not come under the control of α-1-PI and therefore can degrade important organ tissue in individuals with normal levels of α-1-PI? It is believed that the experimental protocol suggested below will clarify the role of α-1-PI in regulating proteolysis. However, it is also probable that the results obtained will still leave us with more questions to ask than those which we have answered.

The objectives of this proposal are to determine the roles of α-1 proteinase inhibitor (α-1-antitrypsin), granulocytic elastase, and other proteases from granulocytes and macrophages, in tissue proteolysis. In particular, we wish to determine what structural differences account for the inability of α-1-PI type ZZ (and also type MZ) individuals to secrete sufficient amounts of inhibitor to combat proteolysis. We would also like to know the mechanism by which the inhibitor normally functions in order to possibly develop synthetic inhibitors. Finally, we would like to determine how the granulocytic and macrophage proteases function in order to again possibly develop better synthetic inhibitors to these enzymes. In this respect we hope to extend studies of this system to an investigation of the development of emphysema in individuals with normal α-1 PI levels. Thus, in individuals who inhale large quantities of particulate matter (i.e. cigarette smoke), are macrophage proteases being released in quantities large enough to overwhelm the normal defense mechanism or does the macrophage actually produce proteases which can digest lung tissue but which are not inhibitable by α-1-PI?

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8. Brief statement of working hypothesis:

It is now believed that the destruction of lung tissue, in type ZZ α-1-PI individuals (and to a lesser extent in type MZ) is due to unrestricted proteolysis by phagocytizing cells due to the absence of α-1-PI. It is suggested that the development of emphysema in individuals with normal α-1-PI may be due to the fact that either the defense mechanism against proteolysis is overwhelmed or that proteases, not inactivated by α-1-PI but capable of degrading lung tissue, are released.

9. Details of experimental design and procedures (append extra pages as necessary)

(see attached pages)

9. DETAILS OF EXPERIMENTAL DESIGN AND PROCEDURES

a) BACKGROUND

Human α-1-proteinase inhibitor (α-1-PI)[1], more commonly called α-1-antitrypsin, is a glycoprotein which has been shown to be responsible for approximately 90% of the trypsin inhibitory capacity of plasma (Heimburger et al., 1971). The broad specificity and high concentration of this protein in plasma and tissue fluids reflect its important role in preventing tissue proteolysis. The high incidence of phagocyte mediated proteolysis of lung tissue observed in some α-1-PI deficient individuals correlates with this function (Laurell and Eriksson, 1963; Fagerhol, 1972). This has resulted in an increased interest in both the properties and function of this protein.

During the past several years many laboratories have published methods for the isolation of human α-1-PI in various states of purification (Moll et al., 1958; Bundy and Mehl, 1959; Schultze et al., 1962; Shamash and Rimon, 1966; Liener et al., 1973; Murthy and Hercz, 1973; Crawford, 1973). Unfortunately, none of the procedures used are desirable because the isolated inhibitor is obtained either in low yield or in a partially inactive form.

As to the actual pathogen in the development of emphysema, it is believed to be proteolytic in nature, based on results such as those obtained by Mass et al., (1972) in which proteases were inhaled as an aerosol and produced emphysema - like lesions in the lung. The type of enzyme suspected is elastolytic in nature and its source is probably granulocytes and/or macrophages. The former type of cell has already been shown to be sequestered in lung bases and these cells can damage the pulmonary capillary membrane (Wilson et al., 1971).

An elastase preparation has been isolated from granulocytes by Janoff and Basch (1971) and, more recently, by Ohlsson (1974). Unfortunately, the purification procedures utilized were not easily followed or resulted in poor yield. At least three forms of elastase were identified, probably resulting from proteolytic cleavage either by autolysis or by other enzymes present during the purification steps.

Recent work on the mechanism of action of α-1-PI has been reported by Moroi and Yamasaki (1974). The results closely parallel those which are given in the current progress report. However, their α-1-PI is not homogenous and they assume 1:1 binding in the interpretation of the data. Nevertheless, the paper is an excellent beginning in understanding α-1-PI inhibition mechanisms.

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b) SPECIFIC AIMS

During the next three years we hope to do the following: 1) Determine at a molecular level, differences between α-1-PI (MM) and α-1-PI (ZZ), 2) outline the mechanism by which α-1-PI inactivates proteolytic activity, particularly granulocytic elastase, 3) begin an investigation of other proteases, such as those in alveolar macrophages, which might be involved in degradation of alveoli and concomitant development of emphysema.

c) METHODS OF PROCEDURE

The discernment of functional differences between α-1-PI (MM) and α-1-PI (ZZ) and the development of a possible mechanism by which α-1-PI inhibits proteolytic activity are overlapping problems. Our method of investigation suggests that of primary importance would be the elucidation of the complete structure, protein and carbohydrate side chain included, of the normal or "wild type" protein (type MM).

Preliminary evidence suggests that major differences do occur in the carbohydrate composition of type MM and type ZZ proteins. However, the protein portion of both molecules may also have minor but important differences. Furthermore, if we are to attempt to establish the inhibition mechanism by which α-1-PI operates, it will be necessary to know something about its primary structure. For example, is there sequence homology between this serum inhibitor and that known to occur in other plant and animal proteinase inhibitors? Do the disulfide bond loops contain the active site of the inhibitor as is known to occur in other well-characterized proteins with this function? What are the cleavage points, if any, when trypsin, chymotrypsin, elastase and other proteinases complex with the inhibitor? We already know (see Progress Report-Part 2a) that α-1-PI does form a complex of MW 78,000 with one mole of trypsin or chymotrypsin and that further addition of protease causes formation of a 60,000 and 48,000 fragment with concomitant disappearance of the 78,000 MW species. How does this occur? What fragments are broken off and where do they come from? Are there two separate active sites on the inhibitor, one of which is not exposed until the 78,000 MW complex is formed? Why does elastase form a complex of MW ≈ 95,000? We feel that structural analysis will aid in elucidating this information. Furthermore, the results may give us some insight as to possible procedures for synthesizing low molecular weight inhibitors which are non-toxic and non-antigenic but whose function is similar to α-1-PI.

We propose to utilize α-1-PI (MM) purified in our laboratory for sequential analysis. We have already begun this study and have cleaved the reduced carboxymethylated protein at methionine residues with cyanogen bromide. We have separated the fragments by gel filtration and ion-exchange chromatography. We intend to sequence the peptides using a Beckman Model 800 sequenator. Where necessary, tryptic and chymotryptic cleavage will also be performed to provide overlapping peptides.

These experiments have now provided us with a C^{14} labelled peptide containing the cysteine residues. We feel, since we have no sequenator at present, that this is the most important part of the protein portion of α-1-PI which we should be examining since it may be at or near the inhibitory site(s). Dr. U. S. Seal, V. A. Hospital, Minneapolis, Minn., has identified the first nine residues in the intact protein for us, using a sequenator, as being Glu-asp-pro-glu-gly-asp-ala-ala-asp. Further analysis cannot be done for us because of reagent costs and a backlog of other samples in Dr. Seal's laboratory.

Carbohydrate side chain analysis of α-1-PI (MM) and α-1-PI (ZZ) is being performed with the assistance of Dr. J. Mendicino, a faculty member in our department who is well-recognized for his work with carbohydrates and glycoproteins. We will use inhibitor isolated by our standard procedure and prepare pronase digests of the two proteins. The glycopeptides will have been purified by ion-exchange chromatography. Each will be sequenced by addition of specific glycosidases followed by quantitative analysis of the sugar or sugar derivatives released. We are also using carbohydrate-containing cyanogen bromide fragments in this study. Our results with the type MM protein indicate to date the release of sialic acid (3 residues), N-acetyl hexosamine, and mannose. Quantitation is, however, not yet complete. We do not plan to use the ZZ protein, which we are accumulating, until the MM structure is conclusively established. This applies not only to carbohydrate analysis but also to peptide fingerprints in establishing any differences in the primary protein structure of the two molecules.

Our further investigations into the mechanism of action of α-1-PI include formation of stable complexes with various proteases, followed by dissociation with hydrazine. The modified inhibitor and other fragments will then be analyzed to see exactly what peptide bonds have been cleaved and where, in the sequence, this is occurring. We hope to establish whether limited proteolysis releases a biologically active fragment with inhibitory properties. This has already been well documented in coagulation processes, complement activations, and zymogen activation. In particular, we would hope to detect two inhibitory sites on -1-PI for elastase, which are both accessible without peptide bond cleavage, to explain the formation of the stable complex of MW ≈ 95,000 which we have detected by SDS gel electrophoresis.

Finally, with regard to α-1-PI, we wish to know whether this protein does combine with thrombin or plasmin. Preliminary experiments indicate that incubation of -1-PI with thrombin does prolong clotting times. Hence, it may regulate the clotting process to some extent. We hope to detect stable complex formation by SDS gel electrophoresis of mixtures of the purified inhibitor and proteases.

Our investigations on the role of elastolytic enzymes in tissue proteolysis and, in particular, with regard to the development of emphysema will continue. To date, no one has attempted to show that granulocytic elastase is truly digesting lung alveoli. We will make antibodies to our human granulocytic elastase and attempt to demonstrate directly or indirectly by fluorescein isothiocyanate treatment the binding of the antibody to elastase in lung tissue. We intend to use normal and diseased tissue in these experiments and, in particular, material obtained from type ZZ individuals who have expired due to emphysema. This latter material is being provided by several hospitals including Mayo Clinic, Johns Hopkins Hospital, and Washington University School of Medicine. Some samples have already been obtained from Dr. R. C. Roberts, Marshfield Clinic. We are currently trying to establish a working relationship with Medical College of Georgia, Augusta, Ga.

Should the results be positive for enzyme localization in the lung, we intend to set up a radioimmunoassay for elastase in serum using I^{125} labelled enzyme. We hope to detect differences in circulating elastase levels which we will try to correlate with various disease states and with α-1-PI phenotypes.

Finally, we plan to examine the properties of granulocytic elastase in some detail in terms of its relationship to elastase(s) from other cell lines (see below) and with regard to its inhibition by other naturally occurring inhibitors. Again, it may be possible, through a detailed delineation of the physical, chemical, and biological properties of the molecule, to develop synthetic inhibitors to this enzyme which might prove useful both therapeutically and for prevention of unrestricted proteolysis.

Since we believe that macrophages and, probably, other cell types as well produce proteolytic enzymes in response to various stimuli we wish to initiate a study of these enzymes. We had planned to harvest such cells by bronchial lavage but this does not seem likely to result in the production of enough material to make enzyme isolation practical. We recently noted a new technique [PNAS 71, 3598 (1974)] for the elicitation of collagenase in the culture fluid of endotoxin-activated peritoneal macrophages. Although we will use macrophages obtained by

bronchial lavage in our preliminary studies, we feel that it would be extremely useful to attempt to culture human alveolar macrophages and to try and stimulate these cells to produce proteases in the culture medium. The media could be harvested and utilized as a source of starting material for enzyme isolation.

Whether we use cells of media for enzyme isolation, we plan to purify as many of the major proteolytic enzymes present and determine whether they are inhibitable by α-1-PI. In particular, we will examine elastolytic proteases. We hope by these methods to determine exactly the role of macrophage proteases in the destruction of lung tissue.

d) SIGNIFICANCE

The proposed research should yield information as to the nature of the defect(s) in α-1-PI synthesis in type ZZ α-1-PI phenotypes. It will allow for the development of routine assays for elastase activity in serum as a possible clinical tool for diagnosis of potential emphysema patients. Finally, the actual significance of macrophages in the lung in terms of their role in lung destruction will be clarified. In particular, this should shed new light on the reasons for the development of emphysema in individuals with normal α-1-PI levels.

It should be noted that, as in the past, a fundamental rule in this laboratory is to work only with highly purified proteins. Crude systems only result in ambiguous results. We feel our approach, strictly at the molecular level, is the only reasonable methodology for truly understanding the roles of α-1-PI, elastase(s), granulocytes, and macrophages in the development of chronic obstructive lung disease.

10. Space and facilities available (when elsewhere than item 2 indicates, state location): The P.I. has a well-equiped laboratory with 1500 sq. ft. of space in the Department of Biochemistry in the Graduate Studies Research Center.

Major Pieces of Equipment directly available include the following:

Beckman Model 120C Amino Acid Analyzer (2)
Zeiss Model PMQ Spectrophotometer
Beckman Model E Analytical Ultracentrifuge
Fraction Collectors (4)
Radiometer pH Meter
Gilson High Voltage Electrophoresis (2)
Mettler Analytical Balance
Mettler Top Loading Balance
Radiometer Conductivity Meter
Sorvall Model RC2B Centrifuge
Table Top Centrifuge (2)

Also available in the department are:

Mass Spectrometers (2)
Novo Computers (2)
Scintillation Counters (4)
Cary Spectrophotometers (3)
Beckman Model L Ultracentrifuge (3)

A complete animal quarters for the preparation of anti-sera is also available.

11. Additional facilities required:

(see attached sheet)

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

11. ADDITIONAL FACILITIES REQUIRED:

Of paramount importance to this project is the purchase of a Beckman Sequenator. The P.I. has sequenced proteins of low molecular weight (6,000) by classical procedures and personally realizes the man-hours involved with a molecule as large as α-1-PI (53,000). There is no doubt that this is an awesome problem to tackle. Nevertheless, it must be accomplished if the true function of α-1-PI is to be realized. A sequenator can ultimately reduce the sequencing process to a realistic time, probably about one year, to complete the structure. Without it, sequencing by hand will probably take three years not only because of the manual manipulations involved but also because about ten to twenty times more protein will be required.

The P.I. realizes that a logical question to be asked is: Who else will use this instrument? What other proteins will be sequenced? In our own laboratory we would also plan to sequence human trypsin and human granulocytic elastase as both are unusual proteases, the former being important, in pancreatic function and the latter, probably, abnormal lung function. We feel that structural analysis will help us to determine why human pancreatic enzymes are so poorly inhibited by naturally occurring inhibitors. It goes without saying that the structure of granulocytic elastase will be very important if we are to later develop synthetic inhibitors against this enzyme. Our long range plans are to examine mast cell proteases and their role in the inflammatory process. Also, little is known of the function of α-1-antichymotrypsin. We hope to purify several of these proteins and probably determine their covalent structure in order to discern the mechanisms by which both the proteases and inhibitor function.

There are many other faculty members who would avail themselves of a sequenator were one purchased. A list of the faculty members and their interests is briefly described:

- Dr. R. Lovins - hapten binding sites on antibodies
- Dr. J. Mendicino - carbohydrate and protein structure of carcinoembryogenic antigen.
- Dr. W. L. Williams - proteases (acrosin) and protease inhibitors in fertilization
- Dr. M. J. Cormier - enzymes involved in vision and bioluminescence
- Dr. H. D. Peck - bacterial electron-transfer proteins
- Dr. J. Brewer - structure and function of enolase
- Dr. L. Ljungdahl - structural differences between thermophilic and mesophilic enzymes with similar biological activities
- Dr. F. Inman - structure of immunoglobulins

The diversity of interests by the above faculty members insures that good use would be made of a sequenator. In particular, it should be pointed out that Dr. Lovins has had considerable experience with sequenators and interfacing them with mass spectrometers for rapid analysis of protein sequences. NSF has recently allotted funds to him as a contribution to the purchase of a sequenator. The current purchase price is about $48,000. Should funds be allotted by The Council for Tobacco Research - U.S.A., the purchase of this instrument would be made from $12,000 from Dr. Lovins, through NSF, $12,000 through this grant from The Council for Tobacco Research - U.S.A., $11,000 from the University of Georgia equipment fund, and the remainder from the Department of Biochemistry.

R: REDACTED MATERIAL

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
James Travis, Associate Professor	50%	REDACTED
D. Johnson, Research Associate	50%	REDACTED
N. Matheson, Research Associate	100%	REDACTED
K. Beatty, Graduate Student	50%	REDACTED
Fringe Benefits		REDACTED
Technical		
J. Bowen, Research Technician	50%	REDACTED
Secretarial help	P.T.	REDACTED
Dishwasher (Student)	P.T.	REDACTED
Fringe Benefits		REDACTED
Sub-Total for A		REDACTED

B. Consumable supplies (by major categories)

Glassware	$2,000
Chemical	2,000
Biological Materials (Blood, macrophages, etc.)	1,000
Sub-Total for B	$5,000

C. Other expenses (itemize)

Travel (Foreign: $700; Domestic: $300)	$1,000
Publication Costs	800
Sub-Total for C	$1,800
Running Total of A + B + C	$28,070

D. Permanent equipment (itemize)

Beckman Model 800 Sequenator	12,000
Digital pH Meter	800
Sub-Total for D	$12,800

E. Indirect costs (15% of A+B+C) E	4,211
Total request	$45,081

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	6,000	2,500	10,000	4,575	45,075
Year 3	R	6,000	2,500	3,000	5,175	42,675

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BUDGET JUSTIFICATION (General)

The justification for $12,000 towards purchase of a sequenator is presented in section 11. During the second year we anticipate the possible need for equipment for tissue culture in order to cultivate alveolar macrophages. Should this not be necessary it will, of course be dropped from the budget. Otherwise, justification will be made in the renewal application for the second year. In the third year a sharp increase in salary is requested to cover the summer salary of the P.I. who will no longer be supported by a Research Career Development Award at that time, his five year fellowship having been completed on June 30, 1977.

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Biochemistry of Chronic Obstructive Lung Disease	CTR - 862R2	66,626	7/1/72 - 6/30/75
Proteolytic Enzymes of the Blood and Pancreas	NIH-HL-14478	123,000	1/1/72 - 12/31/76
Proteolytic Enzymes of the Blood and Pancreas	NIH Career Development Award: 1K04 HL-70264	—	7/1/72 - 6/30/77

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name James Travis

Signature [signature] Date Jan. 15/75

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Responsible officer of institution

Typed Name Robert C. Anderson

Title Vice-President for Research

Signature [signature] Date Jan. 15/75

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CURRICULUM VITAE

NAME: James Travis

REDACTED

RANK: Associate Professor of Biochemistry

EDUCATION:

Year	Degree	Institution
1958	B.S.	University of Manitoba
1960	M.S.	University of Manitoba
1964	Ph.D.	University of Minnesota

TITLE OF MASTERS THESIS: "Separation and Quantitation of Riboflavin and its Derivatives in Mixtures."

TITLE OF DOCTORAL DISSERTATION: "Physico Chemical Properties of Porcine Trypsin." Advisor: Professor I. Eliener

EXPERIENCE:

Vitamin research - University of Manitoba, 1958-60, under supervision of Mr. A. D. Robinson.

Enzyme structure and function - University of Minnesota, 1960-64, under Dr. I. E. Liener.

Enzyme structure and function - Johns Hopkins University, 1964-66, under Dr. W. D. McElroy.

Enzyme structure and function - University of Maryland, 1966-67, as Assistant Professor.

Enzyme structure and function - University of Georgia, 1967-July, 1973, Assistant Professor, July 1972-present, Associate Professor.

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PUBLICATIONS:

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17. M. H. Coan, R. C. Roberts and J. Travis. Human Pancreatic Enzymes. Isolation and Properties of a Major Form of Chymotrypsin. Biochemistry 10, 2711-2717 (1971).

18. M. H. Coan and J. Travis. Interaction of Human Pancreatic Proteases with Naturally Occurring Proteinase Inhibitors. In: Proceedings of the (First) International Research Conference on Proteinase Inhibitors, pp. 294-298. de Gruyter, Berlin, 1971.

19. J. Travis and M. H. Coan. Specificity of Inhibitors for Human Proteases. In: Proceedings of the International Symposium on Proteolysis and Pulmonary Emphysema. Academic Press, New York, N.Y., 1972.

20. D. J. Newman, J. Travis and H. D. Peck. Partial Sequence of a New Type Ferredoxin from a Sulfate Reducing Bacterium. In: Proceedings of the Tenth International Congress of Microbiology, p. 40, 1970.

21. J. Travis. Human Pancreatic Enzymes: Interaction of Human Trypsin with Chicken Ovomucoid. Biochem. Biophys. Res. Comm. 44, 793-796 (1971).

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RANK:

EDUCATION:

Year	Degree	Institution
1961	B.S.	University of Massachusetts
1964	M.A.	University of Massachusetts
1973	Ph.D.	University of Georgia

TITLE OF MASTER'S THESIS: "The Effects of Metabolic Inhibitors on Osmoregulation by Nauplii of Artemia salina at various salinities."

TITLE OF DOCTORAL DISSERTATION: "The Regulation of Fructose 1,6-diphosphatase from Swine Kidney."

EXPERIENCE:

Research Associate - University of Georgia, 1973-present, under Dr. James Travis.

Research Associate - University of Georgia, 1973-73, under Dr. John Lee.

Research Assistant - Ohio State University, 1967-68, under Dr. Joseph Mendicino.

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Research Assistant - University of Massachusetts, 1964-65, under Dr. J. L. Roberts.

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PUBLICATIONS:

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CURRICULUM VITAE

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EDUCATION:

Year	Degree	Institution
1967	B.S.	Memphis State University
1973	Ph.D.	Memphis State University

TITLE OF DOCTORAL DESSERTATION: "Purification and Properties of a Proteolytic Enzyme from Escherichia coli."

EXPERIENCE:

1973-present. Research Associate, University of Georgia

Summers, 1972-73. Instructor of Biochemistry for senior undergraduates and biology graduate students, Memphis State University.

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PUBLICATIONS:

D. A. Johnson and William H. Spell. Purification and Properties of a Protease from Escherichia coli. Fed. Proc. 32, 1593 (1973).

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CTR Grant No. 862R2

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Final Progress Report (July 1/72 - Dec. 31/74)

Biochemistry of Chronic Obstructive Lung Disease

1003546401

PROGRESS REPORT –

This report includes the results obtained from July 1, 1972 through Dec. 31, 1974 or about two and one-half years of the three year project which terminates June 30, 1975.

1. SUMMARY

The overall objectives of the original project were to determine whether the absence of α-1-antitrypsin (heretofore referred to as α-1-proteinase inhibitor or α-1-PI) resulted in abnormal degradation of lung alveoli by uncontrolled proteolysis due to elastase-like enzymes secreted from granulocytes or from the pancreas. The destruction of the alveoli, it was assumed, ultimately resulted in the development of emphysema.

The specific aims were to a) isolate and characterize human pancreatic elastase, b) isolate and characterize elastolytic enzymes from human purulent sputum and human granulocytes and compare each with that from the pancreas, c) to isolate human -1-PI from the two most phenotypically different types of individuals classified as MM and ZZ and study their comparative properties and their ability to interact with pancreatic and leukocytic proteases.

We have accomplished virtually all of the original goals of this project with the exception that we have chosen not to work with purulent sputum since it is far too complicated a mixture of bacterial and human proteins to make any definite conclusions about the sources and importance of enzymes found therein. The leukocytic elastase(s) have been purified, however, and a comparison with a human pancreatic enzyme is being made. The pancreatic enzyme, however, is not a true elastase.

Two forms of α-1-PI, from MM and ZZ plasma, have been isolated and preliminary structural studies indicate major differences in the carbohydrate side chain, although not in sialic acid content. Evidently, this deficiency, which results in the deposition of the abnormal protein (Type ZZ) in the liver, is due to an inborn error in carbohydrate side chain synthesis. Studies on the interaction of the normal inhibitor with several proteases, including leukocytic elastase, indicate that the molecule can bind two moles of enzyme per mole of inhibitor. This would suggest that the real concentration of α-1-PI in plasma is only about one-half of that currently accepted in the literature. The mechanism by which the interaction occurs is not yet known but studies have been initiated in this direction and a working hypothesis is presented below. (Section IIa)

Finally, in order to purify many of the proteins required for several of our studies, new, perhaps revolutionary, techniques for albumin removal from plasma, albumin isolation, protease purification, and general affinity chromatography have been developed. Details on all of the above information are given in subsequent sections.

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2. DETAILED PROGRESS REPORT

a) PURIFICATION OF α-1-PI AND STUDY OF ITS MECHANISM OF ACTION

The initial attempts to purify this protein by affinity chromatography (1) resulted, unfortunately, in the isolation of a pure but inactive protein. Since the major contaminant in the purification was due to albumin, we developed an affinity column containing the conjugate known as blue dextran. This material has a strong affinity for albumin and virtually no affinity for other plasma proteins. As a result we were able to prepare albumin-depleted plasma (2,3) (< 2% albumin left) which we were able to utilize for the subsequent purification of the inhibitor. This was performed by salt fractionation and two ion-exchange chromatography steps (4,5). The resulting material (molecular weight 52,000) had a capacity to bind two moles of trypsin, chymotrypsin, or elastase per mole of inhibitor (6).

Although some studies on the mechanism by which this inhibitor interacted with proteases have been carried out (7), the experiments are far from complete. Our present results indicate that with trypsin (molecular weight 25,000) a simple 1:1 covalently bonded complex of molecular weight 78,000 is first formed. This complex is then acted upon by a second molecule of trypsin to release a polypeptide chain of molecular weight 18,000, which immediately reacts with the trypsin molecule to give a new complex of molecular weight 43,000 (8,9). Such a mechanism involving limited proteolysis is not unusual and occurs in zymogen activation, blood clotting, and complement activations.

The type ZZ inhibitor has been isolated by the same procedure as that for type MM. Preliminary experiments indicate no differences in the amino acid composition of the two proteins but a significant change in carbohydrate composition. This portion of the α-1-PI molecule, which represents 12% of the molecular weight, has substantially less hexosamine content in the type ZZ protein (10). Thus, the apparent reason for α-1-PI (type ZZ) accumulation in liver is due to aberrant side chain synthesis due, presumably, to a defect in a glycosyl transferase in the liver Golgi apparatus where carbohydrate side chain synthesis is known to occur.

b) PURIFICATION OF GRANULOCYTIC ELASTASE

In order to determine if, indeed, elastase enzymes were involved in lung damage, it was necessary to purify this enzyme from both human granulocytes and pancreas.

The isolation of granulocytic elastase was immediately complicated by DNA and RNA binding which tended to precipitate the enzyme. However, by isolating granulocytes using dextran sedimentation and lysing the cells with heparin, we were able to obtain intact granules virtually free of DNA or RNA. Granule lysis in high salt resulted in a very active preparation of crude granulocytic elastase.

The purification of the enzyme(s) has been accomplished by taking into account the inhibitory properties of Trasylol (Kunitz bovine pancreatic trypsin inhibitor) towards granulocytic elastase activity. By preparing an affinity column of Sepharose-trasylol we were able to absorb all of the elastase activity and subsequently desorb by changing pH (from 6.5 to 4.0). This resulted in the isolation of four isoenzymes, all of which had elastolytic activity. A twenty-fold

increase in specific activity occurred in this step. Subsequent ion-exchange chromatography on CM-cellulose resolved the major form of elastase (representing about 70% of the total activity) from the other three forms (11). We believe that proteolysis by other enzymes in granulocytes has caused the formation of the multiple enzymes. We are currently using only the major form for comparative studies which, to date, indicate no similarities between this enzyme and that from pancreas, described below.

The granulocyte elastase (MW 26,000) is rapidly inactivated by α-1-PI and the product has a molecular weight greater than 90,000 indicating direct formation of 2:1 complex. Further studies are in progress as described in the specific aims section of this proposal.

c) ISOLATION AND PROPERTIES OF PANCREATIC PROTEASES

In order to determine whether human pancreatic elastase might play some role in lung digestion or at least be related to the granulocytic enzyme in such a way as to make it useful in model systems, attempts were made to purify the protein. The results were certainly useful but also somewhat unusual. A protein with very similar properties to pancreatic elastase was purified (12,13). This protein, however, could only hydrolyze low molecular weight synthetic substrates as well as denatured proteins. It could not digest elastin. The reasoning behind the results obtained was that the human enzyme was too acidic in nature to bind to elastin despite the fact that it hydrolyzed similar peptide bonds in other proteins. The enzyme was found to be very dissimilar to the granulocytic elastase. However, it is probably very useful in the digestion of hydrophobic proteins in the intestine. A second protease with weak elastolytic activity is present in human pancreas but it is present in insufficient quantities to make isolation feasible.

During the isolation of the new enzyme which we called "protease E", a second form of human trypsin was isolated (14). This anionic protein was similar to cationic trypsin isolated in this laboratory several years ago. However, anionic trypsin is exceedingly unstable and autolyzes rapidly at alkaline pH. At acid pH it is irreversibly denatured. It is doubtful if this enzyme plays an important role in digestion.

d) REMOVAL OF ALBUMIN FROM PLASMA

As stated in section 2a, an important technique used in the purification of α-1-PI involved albumin binding on the insoluble support, sepharose-blue dextran. We have further investigated this phenomenon with very encouraging results. By using directly the chromophore of blue dextran, known as Cibacron Blue F-3GA, we have been able to prepare insoluble supports of this dye attached to sepharose, which can bind up to 30 mg of albumin per ml of packed gel derivative. Thus, on columns 5 x 100 cm we have been able to retain nearly all of the albumin in 500 ml of plasma without saturating the sepharose-dye derivative. A small quantity of albumin, presumably withits dye-binding site blocked because of the attachment of some other metabolite, always passes through the column. This albumin depleted plasma can be utilized for the purification of many plasma proteins usually contaminated by albumin. Other fluids containing albumin, such as cerebrospinal fluid, urine, saliva, tears, etc., could presumably be freed of this protein by

adsorption on sepharose-Cibacron Blue F-3GA and the fluid then utilized for biochemical or physiological studies. We have used other dye derivatives similar to Cibacron Blue F-3GA with identical results.

The albumin can be isolated from these columns by addition of desorbents including K^+, Ca^{++}, Mg^{++}, octanoate, or SCN^-. The latter has proven extremely useful. The column can be re-equilibrated and re-used indefinitely (15). The albumin appears to be pure in form by electrophoresis and has been utilized by Dr. A. Humphries, Medical College of Georgia for stabilizing kidneys prior to transplantation. He has found it superior to any commercial preparations of albumin in that he has been able to keep dog kidneys viable for at least fourteen days by perfusing with our albumin.

e) INHIBITION STUDIES ON PANCREATIC PROTEASES

Because we have now purified six human pancreatic proteases to date in our laboratory, it was felt that a complete study of the inhibition spectra of naturally occurring inhibitors towards these enzymes might prove useful. The results have been submitted for publication (16).

In summary, it was found that human pancreatic proteases are, in general, poorly inactivated by naturally occurring inhibitors, including soybean trypsin inhibitor, Bowman Birk inhibitor, lima bean inhibitor, chicken ovomucoid, Kunitz Pancreatic Trypsin inhibitor, and Kazal Secretory Trypsin inhibitor. Only α-1-PI was a strong inhibitor of all human pancreatic proteases. On the other hand, protease E, the elastase-like protease, was very poorly inhibited by all inhibitors except α-1-PI.

From the above results we were able to determine that Trasylol (Kunitz Pancreatic Trypsin Inhibitor), attached to sepharose by cyanogen bromide activation, should be able to bind human cationic trypsin and one form of chymotrypsin. Acidification of the column would then result in release of the enzymes. By taking advantage of this fact we were able to design a single-step procedure for the isolation of human cationic trypsin and one form of human chymotrypsin, from activated pancreatic extracts, as pure entities separated from each other (17). This procedure completely superceded earlier methods developed in our laboratory.

f) INTERACTION OF α-1-PI WITH HUMAN THROMBIN

In order to determine what other roles α-1-PI might have in regulating proteolysis studies have been initiated on both the clotting and fibrinolytic system.

Although α-1-PI does not instantaneously inhibit thrombin, clotting times are considerably lengthened as incubation of the inhibitor with enzyme is increased. In fact, the slow formation of a complex can be established by SDS gel electrophoresis after 30 min. incubation. These studies are still in progress and are hindered by the necessity to work with highly purified human thrombin. Further studies with this enzyme and with human plasmin are planned and are described in the Specific Aims and Methods of Procedures section of this proposal.

g) NEW TECHNIQUES IN AFFINITY CHROMATOGRAPHY

As a consequence of our work with the Cibacron dyes as affinity chromatography ligands we became interested in the backbone compound from which they are made. This molecule, cyanuric chloride, is highly reactive and can interact with NH_2, OH, and SH groups. Since it has three reactive chloride groups it can be attached to sepharose through one group, and to ligands through the other two chlorides since all three react at different temperatures. Thus, we have now made a variety of sepharose conjugates, heretofore unobtainable by classical cyanogen bromide activation, which whould prove useful in affinity chromatography for the purification of a number of enzymes. The derivatives we have made include sepharose-trasylol which works as well as that described above (section 2e, Ref. 17), sepharose-pectin, which is useful for amylase isolation, sepharose-riboflavin which is being utilized for the isolation of flavoenzymes by others in our department and a number of sepharose dye derivatives which bind the bioluminescent enzymes called luciferases. It should be noted that the attachment of carbohydrates to sepharose in a direct manner has never been described before.

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February 6, 1975

Dr. V. Lisanti
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110 East 59th Street
New York, N. Y. 10022

Dear Dr. Lisanti:

Pursuant to our telephone conversation of a few days ago, I am writing to inform you of some experiments we are attempting in order to rapidly phenotype individuals as potential candidates for the development of emphysema. We are assuming that type ZZ and type MM α-1 proteinase inhibitor (α-1 antitrypsin) phenotypes would be most prone to the disease. Our experimental approach is multi-folded:

a) We have purified type ZZ α-1-PI and we are making antibodies to this protein as well as to type MM on which we have already reported. We hope that the Z protein may have specific antigenic sites not available on the M protein. Hence, it may be possible to isolate directly or by immunoabsorption a monospecific antigen to the Z protein which could be utilized for mass screening of the population by simple immunoprecipitation techniques.

b) We are developing electrophoretic techniques for the direct identification of MZ and ZZ plasma protein patterns. We have several approaches in mind, although we have, at the moment, initiated studies on only a few.

1. We knew that acrylamide slab gel electrophoresis at pH 5.0 gives some separation of the ZZ and MZ protein patterns which can be distinguished. Albumin, however, does cause some interference.

2. We can remove the albumin by the techniques we have developed and perhaps increase the separation to the sensitivity we desire. It is this approach we are favoring at the moment.

3. We have discussed the possibility of using "oblique thinking" and adding trypsin to plasma to form α-1-PI-trypsin complexes which will migrate in distinctively different positions. It may be that due to the charge differences in the various α-1-PI phenotypes we may be able to see interpretable patterns for the various complexes formed. We have not yet started this work.

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We feel that if our techniques work as well as preliminary results indicate, a properly equipped laboratory should be able to analyze at least 48 samples in an eight hour day. The more electrophoresis equipment available, however, the larger the number of samples which could be analyzed.

With the immunodiffusion technique, of course, the sample number is increased immeasurably.

I hope this update will be useful to you and to the Council for Tobacco Research-U.S.A.

Sincerely yours,

James Travis
Associate Professor

JT:pdc

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